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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Photocleavable Flourescent Nucleotides For DNA Sequencing on Chip Constructed by Site-Specific Coupling Chemistry

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 23 ☐ CD(s), Number _____

☐ Drawing(s) *Number of Sheets* _____ ☐ Other (specify) Claims (2 pages)

☐ Application Date Sheet. See 37 CFR 1.76

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☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE _____ Date March 3, 2004

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TELEPHONE 212 278 0400 Docket Number: 0575/72067-PRO

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Jingyue Ju

U.S. Provisional Application No. : Not Yet Known

Filed : Herewith

For : PHOTOCLEAVABLE FLUORESCENT NUCLEOTIDES FOR DNA SEQUENCING ON CHIP CONSTRUCTED BY SITE-SPECIFIC COUPLING CHEMISTRY

1185 Avenue of the Americas
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March 3, 2004

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Photocleavable Fluorescent Nucleotides for DNA Sequencing on a Chip Constructed by Site-Specific Coupling Chemistry

Jingyue Ju

Throughout this application, various publications are referenced. Full bibliographic citations for these publications are found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entirety are hereby incorporated by reference into this application.

Specifically incorporated are the contents of U.S. Patent No. 6,664,079, particularly the names and structures of all nucleotide analogues therein, and all methods for DNA polymerase-based DNA sequencing.

The methods of this invention apply, mutatis mutandis, to the sequencing of RNA.

Abbreviations: DSS, DNA sequencing by synthesis; PC, photocleavable; AFM, atomic force microscopy; Bodipy, 4,4-difluoro-4-bora-3α,4α,-diaza-s-indacene; ROX, 6-carboxy-X-rhodamine; FAM, 5-carboxyfluorescein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

ABSTRACT

DNA sequencing by synthesis (DSS) on a solid surface offers new paradigms to overcome limitations of electrophoresis-based sequencing methods. Here, we report DSS using photocleavable fluorescent nucleotide analogues (dUTP-PC-Bodipy-FL-510, dCTP-PC-Bodipy-650 and dUTP-PC-ROX) on a glass chip constructed by a 1,3-dipolar azide-alkyne cycloaddition coupling chemistry. Each nucleotide analogue consists of a different fluorophore attached to the base through a photocleavable 2-nitrobenzyl linker. We constructed a DNA microarray using the 1,3-dipolar cycloaddition chemistry to site-specifically attach azido-modified DNA onto an alkyne-functionalized glass chip at room temperature under aqueous conditions. Upon verifying that the polymerase reaction could be successfully carried out on the above DNA array, we then performed a sequencing reaction on the chip by using a self-primed DNA template. In the first step, we extended the primer using DNA polymerase and dUTP-PC-Bodipy-FL-510, detected the fluorescent signal from the fluorophore Bodipy-FL-510 and then cleaved the fluorophore using 340 nm UV irradiation. This was followed by extension of the primer with dCTP-PC-Bodipy-650, and the subsequent detection of the fluorescent signal from Bodipy-650 and its photocleavage. The same procedure was also performed using dUTP-PC-ROX. The entire process was repeated 5 times using the three fluorescent nucleotides to identify 7 bases in the DNA template. These results demonstrate that the photocleavable nucleotide analogues can be accurately incorporated into a growing DNA strand during a polymerase reaction on a glass chip, and the fluorophore can be detected and then cleaved with high efficiency using near UV irradiation thereby allowing the continuous identification of the template sequence.

The completion of the Human Genome Project has set the stage for screening genetic mutations to identify disease genes on a genomewide scale (1). Accurate high-throughput methods for resequencing the intron/exon regions of candidate genes are needed in order to explore the complete human genome sequence for disease gene discovery. Recent studies have also demonstrated that an important route for identifying functional elements in the human genome is to sequence the genomes of many species that represent a wide sampling of the evolutionary tree (2). To overcome the limitations of the current sequencing technology based on electrophoresis using laser induced fluorescence detection (3–5), a variety of new DNA sequencing methods have been explored. Such techniques include pyrosequencing (6), mass spectrometry sequencing (7–9), sequencing by hybridization (10), sequence-specific detection of single-stranded DNA using engineered nanopores (11), and sequencing of single DNA molecules (12) and polymerase colonies (13).

Recently, new DNA sequencing approaches based on sequencing by synthesis (DSS) on a solid surface have attracted much attention since they have the potential to offer an efficient method to decipher the genome and screen genetic mutations. However, only limited success with such approaches has been reported thus far. The key requirement for these methods to succeed, is the ability to continuously determine the identity of each nucleotide immediately after its incorporation into a growing DNA strand in a polymerase reaction, and to subsequently remove the reporter signal such as a fluorophore in a rapid and efficient manner after its detection before the incorporation of the next nucleotide. To this end, we have designed a parallel DNA sequencing chip system based on DSS (14). This system involves the construction of a chip with immobilized single-stranded DNA templates that can self-prime for the generation of a complementary DNA strand in a polymerase reaction using four unique fluorescently labeled nucleotide analogues. Each of the 4 nucleotide analogues consists of a fluorophore attached to the base through a photocleavable linker and a small chemically cleavable moiety to cap the 3'-OH group to allow temporary termination of the DNA polymerase reaction after the incorporation of each nucleotide. A four-color fluorescence imager is used to identify the incorporated nucleotide on each spot of the chip. After removing the fluorophore photochemically and cleaving the 3'-OH capping group, the polymerase reaction is allowed to proceed with the incorporation of the next nucleotide analogue and the detection of the

subsequent base on the template sequence. A significant advantage offered by the photochemical cleavage of the fluorescent dye is that no additional chemical reagents are required to be introduced into the system and clean products can be generated with no requirement for subsequent purification. We have previously demonstrated that a 2'-deoxyuridine 5'-triphosphate bearing a fluorophore on its 5 position via a photocleavable 2-nitrobenzyl linker (dUTP-PC-Bodipy-FL-510) is an excellent substrate for the DNA Polymerase *Thermo Sequenase* in a solution-phase DNA extension reaction (15). The fluorophore was shown to be completely cleaved by near-UV irradiation ($\lambda \sim 340$ nm) after its incorporation into a growing DNA strand. Subsequently, Mitra *et al.* also demonstrated the use of the similar photocleavable fluorescent nucleotides for *in situ* DNA sequencing on polymerase colonies (13). These results firmly established the feasibility of using photocleavable fluorescent nucleotide analogues to perform DNA sequencing by synthesis.

Alternative methods were also explored to remove the fluorescence signal from a fluorescent nucleotide in the DSS approach. Braslavsky *et al.* (12) reported the use of photobleaching to eliminate the fluorescent signal from an incorporated nucleotide before the addition of the subsequent nucleotide in polymerase reaction. But in this method the photobleached fluorophores remain with the DNA template and interfere with the polymerase activity for the incorporation of the subsequent nucleotide. Mitra *et al.* (13) reported the utilization of a disulfide group as a chemically cleavable linker to attach a fluorophore to a deoxynucleotide and the use of β-mercaptoethanol to remove the fluorophore after the nucleotide incorporation and detection. However, the disulfide bond can be reversed and destabilized under certain conditions (16, 17).

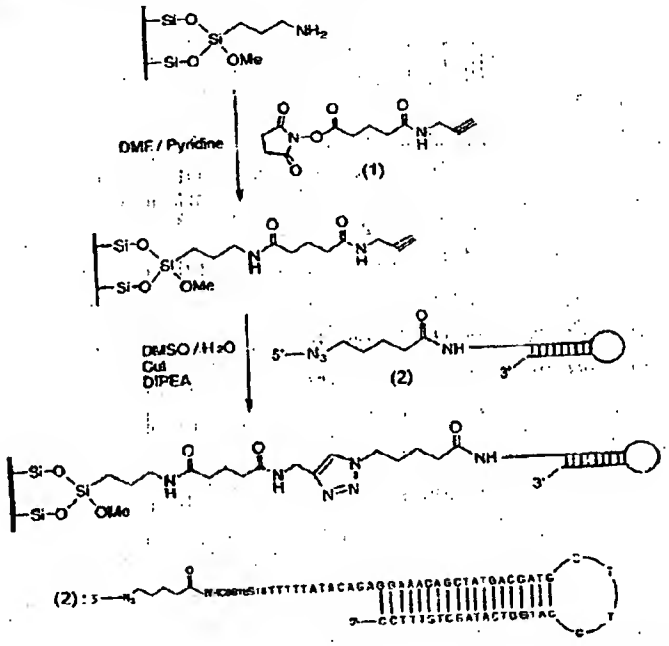
Another crucial requirement for the development of a sequencing approach based on DSS is the construction of a chip with an immobilized self-primed DNA moiety where the primer can not be washed away from the DNA template. The development of a chemoselective coupling chemistry for the immobilization of DNA on a solid surface is essential for accurate gene expression measurement (18), and polymorphism or mutation detection (19, 20). Since covalent coupling chemistries have been shown to typically lead to more stable DNA arrays than non-covalent chemistries, a variety of covalent coupling methods have been utilized for DNA immobilization on a solid surface (21–23). However, further improvement of the coupling chemistry for

immobilizing DNA on a surface is required to achieve high selectivity and coupling efficiency. One ideal property required for the functional groups to be coupled (one from the DNA and the other from the surface) is the stability of the groups in aqueous conditions, which are typically needed to perform the coupling. Previously, we have explored the use of the 1,3-dipolar cycloaddition click chemistry between an azide and alkyne for coupling a fluorophore (FAM) to single-stranded DNA (24). This chemistry was shown to chemoselectively produce FAM-labeled DNA in a quantitative yield under aqueous conditions at 80 °C. Recently, it has been reported that this reaction can be conducted at room temperature in the presence of copper (I) catalyst to produce only 1,4-regioisomeric triazoles (25, 26). Due to its mild reaction conditions, high selectivity and efficiency, this ligation reaction has also been applied for the modification of virus particles with fluorescent dyes (27), cell surface labeling (28), the profiling of enzyme activity in proteomes (29), and the attachment of oligosaccharides to microtiter plate for biological assays (30). We report here the use of this copper (I)-catalyzed [3+2] azide-alkyne cycloaddition to immobilize DNA on a glass chip for DSS using photocleavable fluorescent nucleotides. From the results of this study, we demonstrated that the nucleotide analogues could be successively and accurately incorporated into a growing DNA strand as substrates during a polymerase reaction on a glass chip, and the fluorescent dye could be detected and cleaved with high efficiency using UV irradiation at 340 nm thereby allowing the continuous identification of the template sequence.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 and 300 spectrometers, respectively. High-resolution mass spectrometry (HRMS) data were obtained using a JEOL (Tokyo, Japan) JMS HX 110A mass spectrometer. Mass measurement of DNA was made on a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). A home made contact angle goniometer was used for the water contact angle measurement, and the atomic force microscopy (AFM) images were acquired using a NanoScope multimode scanning probe microscope in the tapping mode. Photolysis was performed at 340 nm (10 nm band pass) using a 450 W high-pressure Xenon lamp (Thermo Oriel, Stratford, CT) in conjunction with a UV interference filter 340 nm (CVI Laser, Albuquerque, NM) with a light intensity of 6 mW/cm^2. The scanned fluorescence images

were obtained using a ScanArray Express scanner (PerkinElmer Life Sciences), which was equipped with four lasers with excitation wavelengths of 488 nm, 543 nm, 594 nm, and 633 nm and emission filters centered at 522 nm, 570 nm, 614 nm and 670 nm, respectively.



Scheme 1. Construction of the DNA chip using 1,3-dipolar azide-alkyne cycloaddition coupling chemistry. A glass chip is first functionalized to contain an alkynyl group, which forms a covalent bond with the 5' azido-modified DNA.

Construction of the DNA chip using 1,3-dipolar azide-alkyne cycloaddition coupling chemistry

The DNA chip was constructed as shown in Scheme 1 involving the following three steps:

1. Synthesis of the crosslinker succinimidyl *N*-propargyl glutariamidate (1). Glutaric anhydride (2.59 g, 22.7 mmol), propargylamine (1.27 g, 23 mmol) and triethylamine (2.33 g, 23 mmol) were dissolved in CH_2Cl_2 (50 mL) and stirred for 12 h at room temperature. After the CH_2Cl_2 solvent was evaporated, the residue was acidified by adding 1 M HCl solution (5 mL). The solvent was removed under vacuum and the crude mixture was purified by silica gel

chromatography ($CHCl_3$:CH_3OH = 5:1, R_f = 0.45) to yield 2.77 g of pure *N*-propargyl glutariamidic acid as a yellow solid (72% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 3.93 (d, 2H), 2.55 (m, 1H), 2.32 (t, 2H), 2.24 (t, 2H), 1.88 (m, 2H). ^{13}C NMR (75.2 MHz, CD_3OD) δ 176.8, 175.0, 80.3, 72.1, 35.8, 34.0, 29.4, 22.1. HRMS (FAB^+) *m/z*: Calcd for $C_8H_{12}O_3N$ ($M+H^+$), 170.0817; Found, 170.0823. To a solution of *N*-propargyl glutariamidic acid (0.40 g, 2.36 mmol) in CH_2Cl_2 (25 mL) was added *N*-hydroxysuccinimide (0.28 g, 2.4 mmol), followed by the addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.46 g, 2.4 mmol) at room temperature. After stirring for 8 h, the mixture was washed with H_2O (2 × 20 mL) and the aqueous layer was extracted with CH_2Cl_2 (30 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to yield 0.47 g of the crosslinker succinimidyl *N*-propargyl glutariamidate **1** as a yellow solid (75 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) 3.99 (q, 2H), 2.81 (s, 4H), 2.64 (t, 2H), 2.29 (t, 2H), 2.19 (t, 1H), 2.05 (m, 2H). ^{13}C NMR (75.2 MHz, $CDCl_3$) δ 171.3, 169.3, 169.0, 168.3, 79.5, 71.3, 34.1, 29.8, 29.5, 29.0, 25.5, 20.6. HRMS (FAB^+) *m/z*: Calcd for $C_{12}H_{15}O_5N_2$ ($M+H^+$), 267.0981; Found, 267.0995.

II. Synthesis of the azido-labeled DNA (2). The amino-labeled hairpin DNA (sequence shown in Scheme 1) was prepared by phosphoramidite chemistry on a DNA synthesizer. The self-primed DNA moiety consisted of a loop with a sequence [G(CTTG)C] and a stem formed using the M13 -28 reverse primer sequence. The stem was followed by a 7-bp sequence at the 5'-end with an 'A' nucleotide inserted alternately (*AGACATA*). Five T nucleotides, two spacer 18 phosphoramidites (S18) and one 5'-amino-modifier C6 phosphoramidite (Glen Research, Sterling, VA) were added subsequently. Azido-labeling of this DNA molecule was achieved by reacting the above 5' amino-modified DNA with succinimidyl 5-azidovalerate in 0.25 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.0) for 12 h at room temperature. The resulting azido-labeled DNA was purified by size-exclusion chromatography and desalted with an oligonucleotide purification cartridge (24). The DNA product was analyzed by MALDI-TOF mass spectrometry (MS) using the amino-modified DNA as an internal standard. The theoretical mass difference between the amino-modified DNA and the azido-labeled DNA was calculated as 125 Da, and the observed mass difference was 119 Da, which is typically within the error range of MALDI-TOF MS measurement for DNA.

III. DNA immobilization on a glass surface. The amino-modified glass slide (Sigma) was cleaned by immersion into a basic solution (dimethylformamide (DMF)/*N,N*-diisopropylethylamine (DIPEA) 9/1 v/v) for 1 h, sonicated for 5 min, washed with DMF and ethanol, and then dried under Ar. The precleaned glass surface was functionalized to contain a terminal alkyne group by immersing it into the alkyne crosslinker solution [20 mM of succinimidyl *N*-propargyl glutaramidate **1** in DMF/pyridine (9/1 v/v)] for 5 h at room temperature. After sonication for 5 min, the glass surface was washed with DMF and ethanol and dried under Ar. The azido-labeled DNA was dissolved in DMSO/H_2O (1/2 v/v) to obtain a 20 μM solution. This DNA solution was then spotted onto the alkynyl-functionalized glass surface in the form of 4-μL drops, followed by the addition of CuI (400 pmol, 5 eq.) and DIPEA (400 pmol, 5 eq.) solution. The glass slide was incubated in a humid chamber at room temperature for 12 h, then washed with deionized water (dH_2O), and SPSC buffer (0.25 M sodium phosphate, 2.5 M NaCl, pH 6.5) extensively for 1 h to remove nonspecifically bound DNAs (31), and finally rinsed with dH_2O and ethanol. AFM and water contact angle measurement were used to characterize the change on the surface after each step in the immobilization process.

Polymerase extension reaction on the DNA chip constructed by 1,3-dipolar azide-alkyne cycloaddition chemistry. The overall procedure for the reaction is shown in Scheme 2. Each area (*a*, *b*, *c*, and *d*) of the chip had two identical spots, each of which was spotted with the azido-modified DNA as described above. The formation of a stable hairpin was ascertained by covering the entire surface with 1X reaction buffer (26 mM Tris-HCl, 6.5 mM $MgCl_2$, pH 9.5), incubating it in a humid chamber at 94 °C for 10 min to dissociate any partial hairpin structure, and then incubating it at 48 °C for 10 min for reannealing. The temperature was then increased to 72 °C to facilitate the primer extension reaction. In the spots *b*, a 0.5 μL solution consisting of 20 pmol of dTTP, 0.5 U of Thermo Sequenase and 1X reaction buffer was added. In the spots *c*, a 0.5 μL solution consisting of 30 pmol of dTTP, 20 pmol of dCTP, 0.5 U of Thermo Sequenase and 1X reaction buffer was added. In the spots *d*, a 0.5 μL solution consisting of 30 pmol of dTTP, 20 pmol of dCTP, 20 pmol of dGTP, 0.5 U of Thermo Sequenase and 1X reaction buffer was added. The glass slide was incubated at 72 °C for 5 min to extend the primer, subsequently washed vigorously with dH_2O, SPSC buffer, 0.1% SDS and ethanol, and dried briefly. Then, a

0.5 μL solution of 10 pmol dye-labeled dNTPs (Perkin Elmer) (Cy3-dUTP for *a*, Cy5-dCTP for *b*, FAM-dGTP for *c*, and Texas-Red-dATP for *d*) and 0.25 U of Thermo Sequenase was added to each spot and a single base extension reaction was performed at 72 °C for 5 min. After the surface was thoroughly washed again with dH_2O, SPSC buffer, 0.1% SDS and ethanol, the fluorescence emission from each spot was detected with a ScanArray Express microarray scanner. The scanning was conducted four times, each time with a different excitation wavelength specific to each of the four fluorophores. The absorption and emission maxima for each of the fluorophores are as follows: FAM (λ_{abs} = 496 nm; λ_{em} = 525 nm); Cy3 (λ_{abs} = 550 nm; λ_{em} = 568 nm); Cy5 (λ_{abs} = 650 nm; λ_{em} = 668 nm); Texas-Red (λ_{abs} = 593 nm; λ_{em} = 612 nm). To investigate the possible nonspecific binding of DNA and free nucleotides on the alkyne-modified surface, amino-modified loop DNA and free dye-labeled nucleotides were spotted on the alkynyl crosslinker modified surface and the above washing and fluorescence detection steps were repeated.

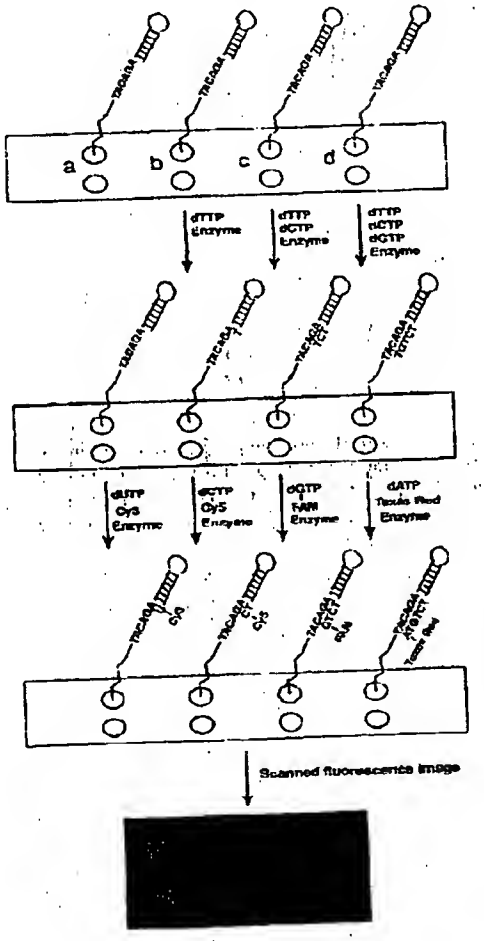
Synthesis of a photocleavable fluorescent nucleotide dCTP-PC-Bodipy-650.

PC-Bodipy-650 was prepared using Bodipy-650 NHS ester (Molecular Probes) and 1-[5-(aminomethyl)-2-nitrophenyl]ethanol following the procedures reported previously for the synthesis of PC-Bodipy-FL-510 (15). ^{1}H NMR (400 MHz, CD_3OD) 8.13 (d, 1H), 7.82 (d, 1H), 7.77 (s, 1H), 7.62 (m, 3H), 7.56 (d, 2H), 7.37 (s, 1H), 7.31 (d, 1H), 7.21 (m, 2H), 7.14 (d, 2H), 7.05 (d, 2H), 6.86 (d, 1H), 5.32 (q, 1H), 4.58 (s, 2H), 4.44 (s, 2H), 3.29 (t, 2H), 2.56 (t, 2H), 1.54 (m, 4H), 1.47 (d, 3H), 1.32 (m, 2H). HRMS (FAB$^+$) *m/z*: Calcd for $C_{38}H_{38}BF_2N_5O_6S$ ($M+H^+$), 741.2611; Found, 741.2583.

PC-Bodipy-650-NHS ester was prepared using PC-Bodipy-650 prepared above and *N,N'*–disuccinimidyl carbonate following the procedures reported previously for the synthesis of PC-Bodipy-FL-510-NHS (15). ^{1}H NMR (400 MHz, CD_3OD) 8.13 (d, 1H), 7.97 (d, 1H), 7.63 (m, 4H), 7.57 (d, 2H), 7.42 (d, 1H), 7.38 (s, 1H), 7.22 (m, 2H), 7.15 (d, 2H), 7.07 (d, 2H), 6.87 (d, 1H), 6.33 (q, 1H), 4.60 (d, 2H), 4.47 (d, 2H), 3.28 (t, 2H), 2.80 (s, 4H), 2.26 (t, 2H), 1.74 (d, 3H), 1.63 (m, 2H), 1.54 (m, 2H), 1.31 (m, 2H). HRMS (FAB$^+$) *m/z*: Calcd for $C_{43}H_{41}BF_2N_6O_{10}S$ ($M+H^+$), 882.2674; Found, 882.2697.

dCTP-PC-Bodipy-650 was synthesized from the coupling reaction between PC-Bodipy-650-NHS ester and 5-aminoallyl-2'-deoxycytidine-5'-triphosphate (dCTP-NH_2, Trilink-

biotechnologies, CA). To a solution of dCTP-NH_2 (1 mg, 2 μmol) in 300 μL 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 8.5) was added PC-Bodipy-650-NHS ester (3 mg, 3.5 μmol) in 300 μL acetonitrile and then stirred at room temperature for 5 h. Preparative thin layer



Scheme 2. Polymerase extension reaction using four fluorescent nucleotides on a DNA chip constructed using the 1,3-dipolar cycloaddition chemistry. Each of the 4 colors in the fluorescence image matched with the fluorescence emssions from the 4 fluorophores used to label the corresponding nucleotides (Green, Cy3; Red, Cy5; Blue, FAM; Orange, Texas Red).

chromatography was used to remove the unreacted PC-Bodipy-650-NHS ($CHCl_3$:CH_3OH = 4:1) and the fractions containing dCTP-PC-Bodipy-650 were collected and purified further by reverse-phase HPLC using similar conditions as described previously (15). The identity of the purified compound dCTP-PC-Bodipy-650 was confirmed by using it to generate a DNA extension product 5'-TCAAGGACGTACCCGCC(*PC-Bodipy-650*)G-biotin-3', which was characterized by MALDI-TOF MS (Calcd, 6669; Found, 6668). The detailed procedure is described in the next section.

Synthesis of dUTP-PC-ROX

dUTP-PC-ROX was synthesized and characterized following a similar procedure as described above (MALDI-TOF MS characterization of the DNA extension product 5'-AGAGGATCCAACCGAGACU(*PC-ROX*)G-biotin-3' containing dUTP-PC-ROX: Calcd, 7308; Found, 7307).

Characterization of the photocleavable nucleotide analogues using a DNA extension reaction and photocleavage studies.

DNA-extension reaction using dUTP-PC-ROX. The reaction was conducted using dUTP-PC-ROX, ddGTP-Biotin (Perkin-Elmer), primer (5'-AGAGGATCCAACCGAGAC-3'), and a synthetic DNA template (100 bp) corresponding to a portion of exon 7 of the human p53 gene following a similar procedure as reported previously (15). The two nucleotides in the template immediately adjacent to the annealing site of the primer were 5'-CA-3'. Thus, the extension reaction would be terminated after extension by only two bases (U-PC-ROX and then G) to generate the DNA fragment 5'–U(PC-ROX)G–Biotin (Fig. 2). The reaction mixture consisted of 20 pmol of template, 40 pmol of primer, 40 pmol of dUTP-PC-ROX, 60 pmol of ddGTP-biotin, 2 μL of 10x reaction buffer, and 2 units of Thermo Sequenase DNA polymerase in a total volume of 20 μL. The reaction consisted of 30 cycles of 94 °C for 20 sec, 48 °C for 30 sec, and 60 °C for 60 sec. Subsequently, the extension product was purified by polyacrylamide gel electrophoresis and streptavidin-coated solid phase capture. The purified DNA extension product was collected and freeze-dried for MALDI-TOF MS measurement and photolysis using the same procedure as described (15).

DNA extension reaction using dCTP-PC-Bodipy-650. A DNA extension reaction was conducted using dCTP-PC-Bodipy-650, ddGTP-biotin (Perkin-Elmer), primer (5'-TCAAGGACGT-ACCCGC-3'), and the same template as above. In this case, the two nucleotides in the template immediately adjacent to the primer annealing site were 5'-CG-3'. Thus, the extension reaction was terminated after extension by only two bases (C-PC-Bodipy-650 and then G) to generate the DNA fragment. The reaction conditions were the same as above, except 40 pmol of dCTP-PC-Bodipy-650 was used instead of dUTP-PC-ROX. The downstream purification, subsequent MALDI-TOF MS measurement and photolysis were performed in the same manner as above.

DNA sequencing by synthesis on a chip. One microliter of a solution consisting of dUTP-PC-Bodipy-FL-510 (20 pmol), Thermo Sequenase (2 U) and 1X reaction buffer was spotted on the area of the glass surface immobilized with the self-primed DNA, and the nucleotide analogue was allowed to incorporate into the primer at 72 °C for 5 min. After washing with dH_2O, SPSC buffer, 0.1% SDS, dH_2O and ethanol, the surface was scanned with the ScanArray Express microarray scanner to detect the fluorescence signal. To perform the photocleavage reaction, the glass chip was irradiated by UV light (λ~340 nm) for 10 min in acetonitrile/water (1/1 v/v) solution. After washing with dH_2O, SPSC buffer, 0.1% SDS and ethanol, the surface was scanned again to compare the intensity of fluorescence after photocleavage with the original fluorescence intensity. This was followed by the incorporation of dCTP-PC-Bodipy-650, with the subsequent washing, fluorescence detection and photocleavage processes performed as described above. The next three steps involved primer extension first by dUTP-PC-ROX, then by dGTP and dUTP-PC-Bodipy-FL-510 and finally by dATP and dUTP-PC-ROX, with the washing, detection and photocleavage steps repeated between every successive incorporation. The absorption and emission maxima for each fluorophore used to construct the photocleavable fluorescent nucleotides are as follows: Bodipy-FL-510 (λ_{abs} = 502 nm; λ_{em} = 510 nm); Bodipy-650 (λ_{abs} = 630 nm; λ_{em} = 650 nm); ROX (λ_{abs} = 575 nm; λ_{em} = 602 nm).

Results and Discussion

The DNA chip was constructed as shown in Scheme 1 using the 1,3-dipolar cycloaddition coupling chemistry to attach the azido-labeled DNA onto the alkyne-modified glass surface in the presence of a Cu(I) catalyst. This cycloaddition is carried out under mild reaction conditions

in an aqueous solution with high selectivity and efficiency. In addition, the two functional groups (azido and alkynyl) to be coupled are very stable in aqueous conditions. Thus, the 1,3-dipolar cycloaddition coupling chemistry provides an ideal coupling reaction to immobilize DNA covalently on a surface to construct a DNA chip. During the immobilization process, the change in topography of the solid surface was monitored and characterized by AFM (Fig. S1, Supporting information). The AFM data indicate that the immobilization of the alkynyl crosslinker led to an increase in the grain size from 0.0035 μm^2 to 0.0079 μm^2 and a small change in the topography. The AFM image of the immobilized DNA layer showed many peaks distributed over the surface. Each peak shows the immobilized DNA molecules with a height range between 3.5 nm and 8.0 nm (the average value is 5.9 nm). Because the predicted length of a fully stretched looped DNA shown in Scheme 1 is about 20 nm, these data indicate that the immobilized DNA molecules are more likely folded. To further confirm DNA attachment on the surface, contact angle measurement with dH_2O drops was performed on the amino-modified surface, alkyne-modified surface and the surface with immobilized DNA using the same substrate. The contact angle of the alkyno-modified surface (47°±1°) was similar to that of the amino-modified surface (49°±1°), whereas the contact angle of the surface with immobilized DNA (34°±2°) predictably decreased drastically due to the hydrophilicity of DNA.

To evaluate the functionality, accessibility and stability of the surface bound DNA, a polymerase extension reaction was carried out using conventional dNTPs (Scheme 2). The DNA moiety contained a hairpin structure that formed an entity capable of self-priming in a polymerase reaction. The self-primed DNA moiety formed by the specific loop sequence [G(CTTG)C] has been shown to be thermally stable with a melting temperature of 86 °C (32). Due to the specificity of self-priming, the possibility of mispriming was reduced. The sequence beyond the priming site was chosen as AGACATA, consisting of 'A' nucleotides alternating with other nucleotides. This sequence was followed by two spacers and an amino-linker that facilitated the attachment of DNA on the glass surface by reducing the effect of steric hindrance. The azido-modified loop DNA was spotted on the chip surface at areas designated as *a*, *b*, *c* and *d* (two spots were made for each label to demonstrate the reproducibility of the data). After DNA immobilization, the glass chip was incubated at 94 °C for 10 min and 48 °C for 10 min to ensure the complete denaturation and subsequent renaturation of the hairpin structure. In the first step of

the extension reaction, unmodified dNTPs (none for spots *a*, dTTP for spots *b*, dTTP and dCTP for spots *c*, and dTTP, dCTP, dGTP for spots *d*) and Thermo Sequenase DNA Polymerase were added to each spot in accordance to the 7-bp sequence mentioned above. After the primer extension reaction, the four spots *a*, *b*, *c* and *d* each had the primer extended by zero, one, three and five bases, generating DNA moieties with free A, G, C, and T bases immediately after the priming site on the template respectively. Each of four dye-labeled dNTPs (Cy3-dUTP for *a*, Cy5-dCTP for *b*, FAM-dGTP for *c*, and Texas Red-dATP for *d*) was then added to the corresponding spot to allow the correct incorporation of a specific fluorescent nucleotide complementary to the corresponding base on the template. As shown in Scheme 2, four distinct fluorescence signals with different colors were generated by using the ScanArray scanner, matching the fluorescence emission produced by the fluorescent nucleotide incorporated on that particular spot. These results confirmed that the fluorescent nucleotides were accurately incorporated in a base-specific manner into the self-primed DNA after primer extension, and the synthesized DNA chip was thermally stable at high temperature. This established the feasibility of carrying out DNA extension reaction on the chip constructed by 1,3-dipolar azide-alkyne cycloaddition coupling chemistry.

We also investigated the nonspecific binding of DNA and free nucleotides on the chip as negative controls. Amino-modified DNA (DNA without azido group) was spotted onto the alkyne-modified surface, and the washing and primer extension reactions were performed in the exact same manner as described above. The comparison of fluorescent intensities from the unlabeled DNA with those from the azido-labeled DNA showed that the non-covalently bound DNA contributed to less than 10% of the fluorescence signal, thereby indicating that the nonspecifically bound DNA was efficiently removed during the washing step. Similarly, the intensity of free dye-labeled dNTPs bound on the alkyne crosslinker surface after washing was shown to contribute to less than 1% of the fluorescence signal. These results proved that the majority of the fluorescence signal was obtained specifically from the covalently bound DNA and therefore established the high efficiency and specificity of the 1,3-dipolar cycloaddition chemistry in covalently attaching DNA on the solid surface.

To demonstrate the feasibility of carrying out DSS on the chip, three fluorescent deoxynucleotide analogues (dUTP-PC-Bodipy-FL-510, dCTP-PC-Bodipy-650 and dUTP-PC-ROX) (Fig. 1) were synthesized and used to perform a sequencing reaction on the solid surface. The nucleotide analogues were synthesized by a similar synthetic method as reported before (15). These nucleotide analogues have different fluorescent dyes attached to the 5 position of the base (U/C) through a photocleavable 2-nitrobenzyl linker. Previously, we have demonstrated that a nucleotide analogue dUTP-PC-Bodipy-FL-510 can be faithfully incorporated by DNA polymerase into a growing DNA strand in a solution-phase polymerase reaction, and that its incorporation does not inhibit the addition of the subsequent nucleotide. We have also shown that near-UV irradiation leads to the efficient release of the fluorophore, ensuring that the previous fluorescent signal does not leave any residue that could otherwise interfere with the detection of the subsequent nucleotide (15). Here, we characterized the other two nucleotide analogues dCTP-PC-Bodipy-650 and dUTP-PC-ROX in a similar manner and have included the data for dUTP-PC-ROX as an example. We performed a DNA extension reaction using dUTP-PC-ROX, ddGTP-biotin, and a synthetic template (100 bp) corresponding to a portion of the exon 7 of the human *p53* gene to generate a DNA extension product 5'–U(PC-ROX)G-Biotin

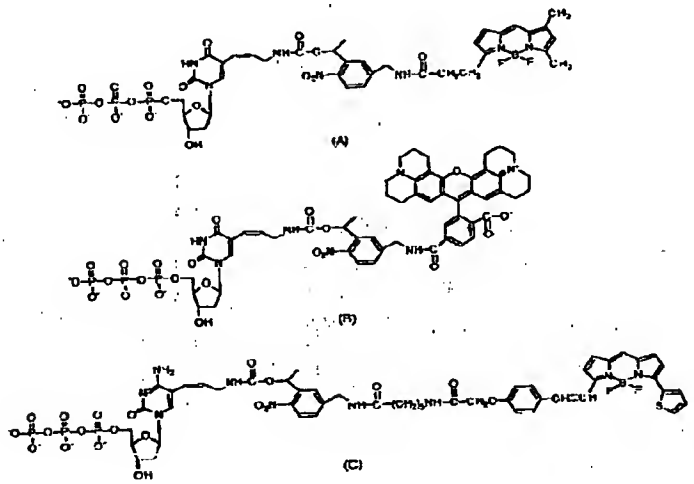


Fig. 1. Structures of dUTP-PC-Bodipy-FL-510 (A), dUTP-PC-ROX (B) and dCTP-PC-Bodipy-650 (C).

(Fig. 2). The purified product 5'–U(PC-ROX)G-Biotin was analyzed by MALDI-TOF MS as shown in Fig. 3 (A), where a strong peak corresponding to 5'–U(PC-ROX)G-Biotin (m/z 7307, Found; 7308, Calcd) is observed. A small peak at m/z 6570 that corresponds to the photocleaved fragment 5'–UG-Biotin (m/z 6569, Calcd) was also observed. This is due to the photocleavage induced by the nitrogen laser pulse (337 nm) used for ionization in MALDI-TOF MS analysis. These results indicated that dUTP-PC-ROX was efficiently incorporated into the growing DNA strand by a DNA polymerase and that its incorporation does not hinder the addition of the subsequent nucleotide.

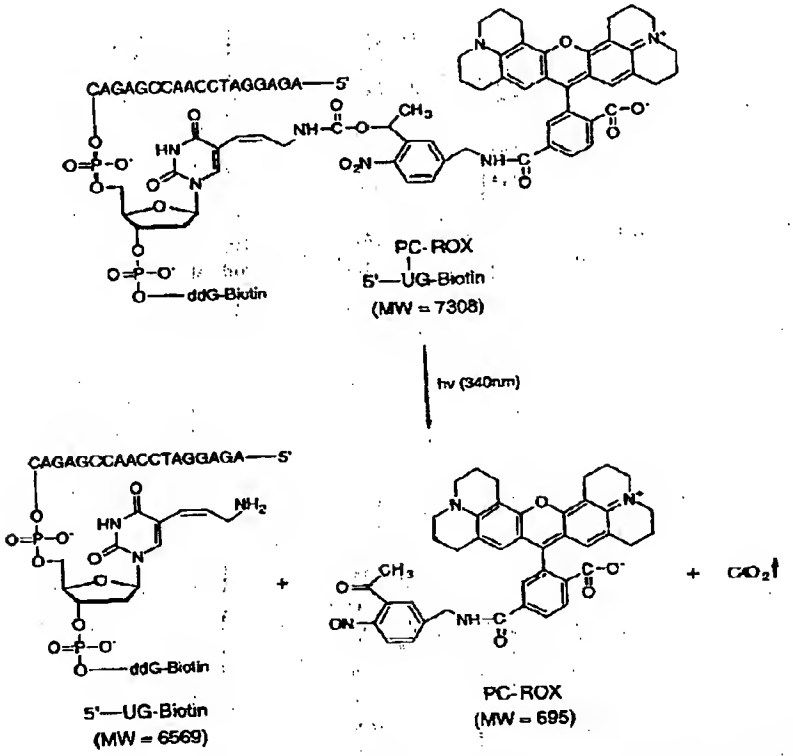


Fig. 2. DNA product *5'–U(PC-ROX)G-Biotin* formed by incorporating a dUTP-PC-ROX into a primer in a polymerase reaction and its photocleavage producing a DNA fragment *5'–UG-Biotin* and *PC-ROX*.

Complete photocleavage of the fluorophore, ROX, from the DNA extension product 5'–U(PC-ROX)G-Biotin is essential for the successful application of dUTP-PC-ROX in the DSS approach. We investigated the photocleavage efficiency of the DNA extension product by MALDI-TOF mass spectrometry. Three minutes of UV irradiation at 340 nm of the solution

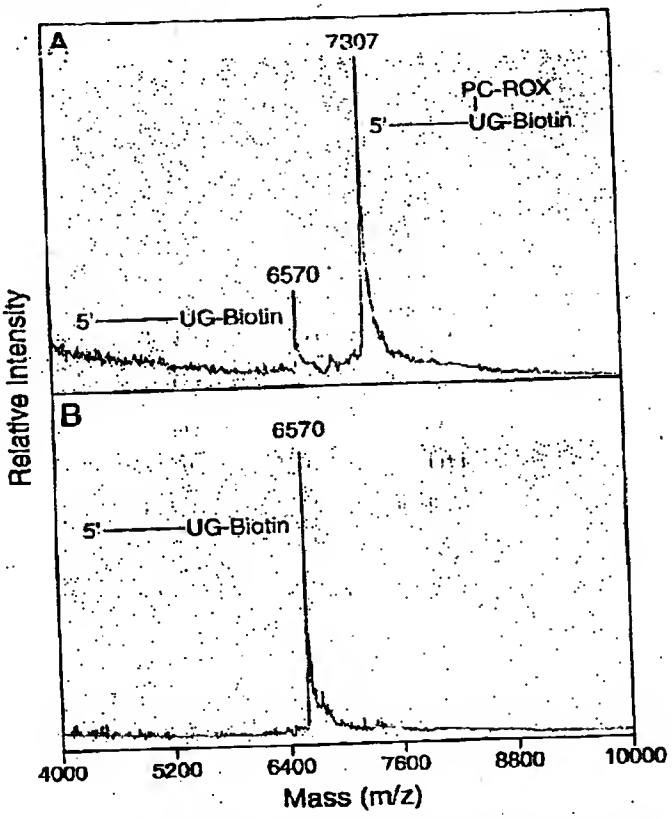


Fig. 3. MALDI-TOF MS spectra of the DNA extension product *5'–U(PC-ROX)G-Biotin* (m/z: found 7307; calcd. 7308) obtained using dUTP-PC-ROX and its photocleavage product *5'–UG-Biotin* (m/z: found, 6570; calcd. 6569). (A) Without irradiation. (B) After 3 min of irradiation of the *5'–U(PC-ROX)G-Biotin* (λ_{irr} ~ 340 nm).

containing the extension product 5'–U(PC-ROX)G-Biotin eliminated the corresponding peak (m/z 7307) in the MALDI-TOF mass spectrum but significantly increased the signal of the photocleaved fragment 5'–UG-Biotin (m/z 6570) as shown in Fig. 3 (B). These data confirm that

the fluorescent dye is completely released from the DNA template in solution within 3 min of UV light irradiation at 340 nm.

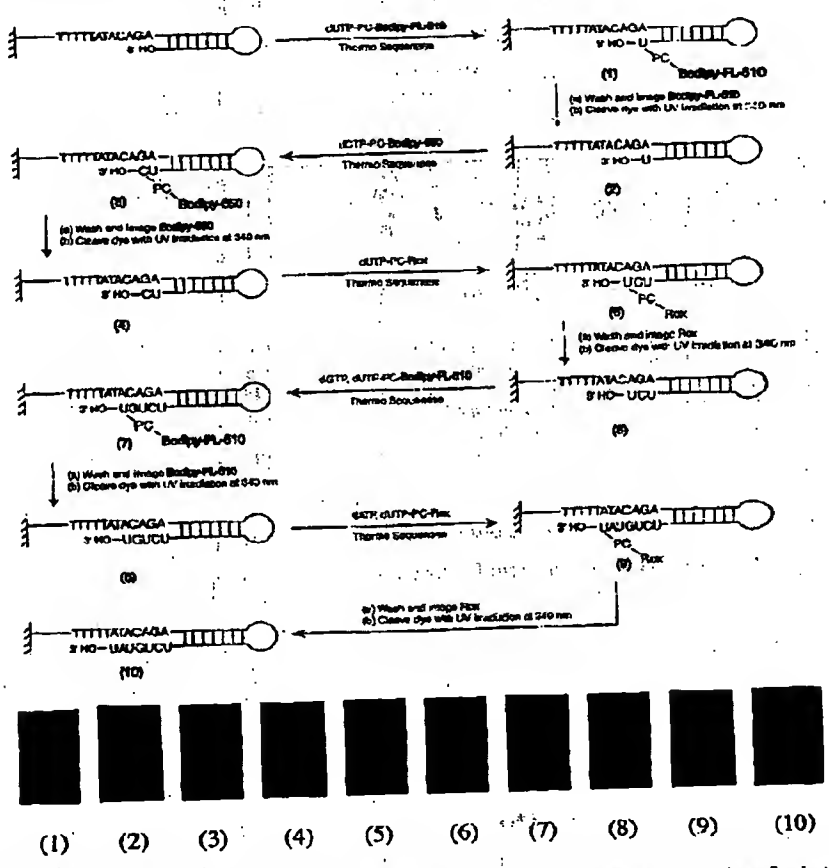


Fig. 4. Schematic representation of DNA sequencing by synthesis (DSS) on a chip using 3 photocleavable fluorescent nucleotides (top) and the scanned fluorescence images for each step of DSS on a chip (bottom). (1) Incorporation of dUTP-PC-Bodipy-FL-510; (2) Photocleavage of PC-Bodipy-FL-510; (3) Incorporation of dCTP-PC-Bodipy-650; (4) Photocleavage of PC-Bodipy-650; (5) Incorporation of dUTP-PC-ROX; (6) Photocleavage of PC-ROX; (7) Incorporation of dGTP and dUTP-PC-Bodipy-FL-510; (8) Photocleavage of PC-Bodipy-FL-510; (9) Incorporation of dATP and dUTP-PC-ROX; (10) Photocleavage of PC-ROX.

The procedure for DNA sequencing by synthesis on the chip is illustrated in Fig. 4 (top) and the corresponding fluorescence image for each step is shown in Fig. 4 (Bottom). The self-primed

DNA was first extended using DNA polymerase and dUTP-PC-Bodipy-FL-510, complementary to the 'A' on the template. After vigorous washing, the extension of the primer was confirmed by observing a blue signal (the emission of Bodipy-FL-510) in the scanner with 488 nm excitation, which is produced only if dUTP-PC-Bodipy-FL-510 is successfully incorporated into the DNA. After washing and detection, near-UV irradiation was applied to cleave the fluorophore from the DNA, which should in principle remove the blue signal efficiently. The scanned fluorescence images obtained from our experiments were shown in Fig. 4 (Bottom). After the first incorporation by dUTP-PC-Bodipy-FL-510, a blue signal was detected [(1) in Fig. 4]. This blue signal from Bodipy-FL-510 was almost completely removed after 10 min of near-UV irradiation, producing an image with fluorescence levels close to background [(2) in Fig. 4]. The integrated fluorescence intensity on the spot, obtained using the scanner software, indicated that more than 97% of the original fluorescence signal was removed by photocleavage. To illustrate the continuity of this reaction on the solid surface, we next used dCTP-PC-Bodipy-650, complementary to the 'G' on the template, to produce a red signal (emission from Bodipy-650) at the same position on the chip in the scanner with 633 nm excitation [(3) in Fig. 4], which was again almost entirely removed after photocleavage [(4) in Fig. 4]. We repeated this process three more times first using dUTP-PC-ROX that gave an orange signal (emission from ROX with 594 nm excitation) after incorporation [(5) in Fig. 4], then dGTP with dUTP-PC-Bodipy-FL-510 [(7) in Fig. 4] and finally dATP with dUTP-PC-ROX [(9) in Fig. 4]. The fluorescence intensity obtained after 5 cycles of incorporation indicated that the incorporation efficiency was about 90%. In this manner, we identified a 7-nucleotide sequence in the DNA template by alternate incorporation, detection and photocleavage using the three photocleavable nucleotide analogues.

To make sure that the observed fluorescence signal is not due to fluorescent photocleavable nucleotides noncovalently bound to DNA, we carried out a control experiment, in which we incubated the self-primed DNA moiety with dUTP-PC-Bodipy-FL-510 after step 10 [(10) in Fig. 4] in the absence of DNA polymerase. After performing the washing steps as described before, the fluorescence intensity observed was less than 3% of that seen after step 1 [(1) in Fig. 4] proving that the strong fluorescence signal is only observed when a fluorescent nucleotide complementary to the free base in the DNA template is specifically incorporated into the DNA strand. The above results firmly established that the fluorescent photocleavable nucleotides can

be faithfully incorporated by a DNA polymerase into a growing DNA strand on a solid surface, and by alternately using photocleavage with primer extension, the polymerase reaction can be continuously carried out on the chip to sequence DNA.

In conclusion, we have successfully constructed a DNA chip using the 1,3-dipolar azide-alkyne cycloaddition reaction, offering a novel chemistry to covalently and chemoselectively immobilize DNA on the solid surface. Furthermore, we have demonstrated the feasibility of performing the DNA polymerase reaction continuously on the chip using photocleavable fluorescent nucleotides. Primer extension and photocleavage reactions were successfully performed on the chip, confirming that DNA sequencing by synthesis can be carried out on the solid surface.

We are currently developing four nucleotide analogues (A, C, G and T), each consisting of a unique fluorescent dye attached to the base through a photocleavable linker and a capped 3'-OH group. The goal is to discover nucleotide analogues that will be able to incorporate into a growing DNA strand on a chip as temporary terminators. The subsequent identification of the fluorophore using a four-color fluorescence imager will reveal the identity of the incorporated nucleotide on each spot of the chip. Upon photochemically removing the fluorophore and regenerating the 3'-OH group, the polymerase reaction will proceed to incorporate the next nucleotide analogue and detect the next base, and so on until the entire template will be sequenced. Since the photocleavage is performed in each cycle of nucleotide addition, any residual fluorescent dye that is left over from the previous cycle will be further photocleaved, ensuring a constant low background. The covalent and chemoselective 1,3-dipolar cycloaddition coupling chemistry for immobilizing DNA on a surface and the library of photocleavable fluorescent nucleotides will also facilitate the development of single molecule DNA sequencing and digital gene expression analysis approaches. With the current efficiency of nucleotide incorporation and photocleavage, we expect to be able to sequence at least 25 bases per spot on the chip, which is sufficient for SNP detection and for gene expression measurement. By reducing the photocleavage time and improving the read length and incorporation efficiency, this approach can potentially be developed into a high-throughput DNA analysis system for whole genome sequencing, SNP detection and applications in pharmacogenetics.

The AFM images of the glass chip surface are shown in Fig. S1. The AFM data indicate that the immobilization of the alkynyl crosslinker led to an increase in the grain size from a diameter of about 80 nm to 140 nm and a small change in the topography. The AFM image of the immobilized DNA layer showed many peaks distributed over the surface, indicating that the immobilized DNA rises over the surface instead of lying down. Each peak shows the immobilized DNA molecules with a height range between 3.5 nm and 8.0 nm (the average value is 5.9 nm). Because the predicted length of a fully stretched looped DNA shown in Scheme 1 is about 20 nm, these data indicate that the immobilized DNA molecules are more likely folded. The peaks are approximately 100 nm in diameter, significantly larger than the space occupied by a single DNA molecule in the particular folded configuration, suggesting that each peak in the AFM image of the DNA-modified surface in Fig. S1(c) corresponds to an aggregate of a number of DNA molecules, which produce the detected fluorescence signals on incorporation of the corresponding fluorescent nucleotides in a polymerase reaction.

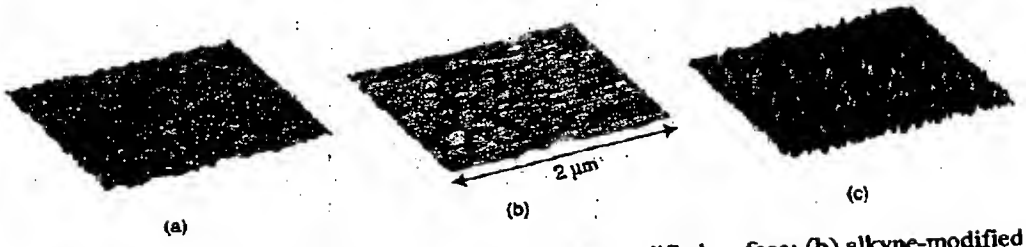


Fig. S1. Surface topographic map by AFM; (a) amino-modified surface; (b) alkyne-modified surface; (c) surface with immobilized DNA. Scan size was 2 μm × 2 μm and the height scale was 20 nm. AFM was carried out in air with a typical tapping frequency of 273 KHz and a nominal scanning rate of 1 Hz.

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What is claimed is:

1. A method for determining the sequence of about 1000 or fewer copies of the same DNA, wherein (i) each DNA is bound to a solid substrate via 1,3-dipolar azide-alkyne cycloaddition chemistry and (ii) each DNA comprises a self-priming moiety, comprising the following steps for each nucleic acid residue of the DNA to be sequenced:

   (a) contacting the bound DNAs with DNA polymerase and four photocleavable fluorescent nucleotide analogues under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (i) the nucleotide analogues consist of an analogue of G, an analogue of C, an analogue of T and an analogue of A, and (ii) each of the four analogues has a pre-determined fluorescence wavelength which is different than the fluorescence wavelengths of the other three analogues, so that a nucleotide analogue complementary to the residue being sequenced is joined to the DNA by the DNA polymerase;

   (b) removing unjoined analogues;

   (c) photocleaving the fluorescent moiety from the joined analogue; and

   (d) determining the identity of the photocleaved moiety,

   thereby determining the sequence of the DNA.

2. The method of claim 1, wherein the solid substrate is glass or quartz.

3. The method of claim 1, wherein the number of DNAs sequenced is fewer than 100.

4. The method of claim 1, wherein the number of DNAs sequenced is fewer than 20.

5. The method of claim 1, wherein the number of DNAs sequenced is fewer than five.

6. The method of claim 1, wherein the number of DNAs sequenced is one.